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Notes:

Oligonucleotide that binds nuclear factor NF- κ B acts as a lymphoid-specific and inducible enhancer element

(immunoglobulin κ light chain gene/transfection)

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ABSTRACT The immunoglobulin κ light chain gene contains a lymphoid-specific enhancer that includes several short protein-binding sequences. The sequence that binds the nuclear factor NF- κ B was tested for its ability to act independently as an enhancer element by inserting it into test plasmids containing the chloramphenicol acetyltransferase gene. When analyzed for activity by transient transfection into lymphoid and nonlymphoid cells, a single copy of the NF- κ B binding site could act as a tissue-specific upstream activating element. Two copies (dimer) showed 10-fold higher activity than did one copy and could act as an enhancer element 2.5 kilobases downstream of the transcriptional start site. The enhancer activity of this sequence was correlated with the presence of the cognate binding protein, NF- κ B. This sequence acted as an inducible enhancer under conditions that induce NF- κ B binding activity. Thus, the NF- κ B binding site acts by itself as a tissue-specific and inducible enhancer element, and two copies show cooperative interaction.

The specific expression of immunoglobulin κ light chain genes in mature B cells is regulated by multiple sequences within the promoter and enhancer regions (1-3). The lymphoid-specific enhancer of the κ light chain gene contains several short sequence elements that bind trans-acting factors (4). Within the enhancer, there are three copies of a sequence homologous to the consensus sequence CAGGTG-GC, or E motif, originally described by Ephrussi *et al.* (5) as protein binding sites in the immunoglobulin heavy chain enhancer. In addition, the κ light chain gene enhancer (" κ enhancer") contains the sequence GGGACTTTC, called the B or κ B site, which binds the nuclear factor NF- κ B (4). Each of these sites has been shown by mutational analysis to be important to the functional activity of the κ enhancer (6). Clustered mutations in the κ B site completely abolish enhancer function identifying this site as crucial to κ enhancer activity.

The DNA-binding protein NF- κ B is found constitutively only in mature B cells that transcribe κ light chain genes. In pre-B-cells stimulated with lipopolysaccharide (LPS), the activation of κ light chain gene transcription is correlated with an induction of the NF- κ B DNA binding activity by a posttranslational mechanism (7). Thus, the NF- κ B protein is important in the activation of κ light chain gene transcription during B-lymphocyte development.

Interestingly, NF- κ B plays a role in the activation of other genes as well. NF- κ B binding sequences are found in the human immunodeficiency virus (HIV) enhancer (8, 9); the simian virus 40 (SV40) enhancer (10), and the cytomegalovirus enhancer (11) and upstream of major histocompatibility complex class I genes (12, 13). The NF- κ B DNA-binding protein can be induced in T cells or HeLa cells by treatment

with phorbol ester (7). In the case of the HIV enhancer, which is activated in T cells, two tandem copies of the NF- κ B binding site are present in the long terminal repeat region. Mutation of these NF- κ B binding sequences prevents the activation of the HIV enhancer in T cells stimulated with phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) (9). This suggests that the nuclear factor NF- κ B is important to the activation of the HIV enhancer in T cells. The possible role of NF- κ B in regulating gene expression in nonlymphoid cells has not been established.

To better understand the mechanism of tissue-specific enhancer activity, we tested whether the NF- κ B binding site could act independently as an enhancer element. We found that a single copy of the κ B site acts as an invertible upstream activating element. Dimers of the κ B site show 10-fold higher activity and function as an enhancer element 2.5 kilobases (kb) downstream of the start of transcription. The enhancer activity of the κ B-containing oligonucleotide was highest in mature B cells but could be induced in pre-B-cells treated with LPS or in T cells treated with PMA and PHA. We conclude that the sequence that binds NF- κ B can act by itself as a tissue-specific and inducible enhancer element.

MATERIALS AND METHODS

Plasmid Constructions. Plasmids were derived from the Δ -71-c-fos-CAT plasmids described by Gilman *et al.* (14). This plasmid was modified by inserting an *Xho* I linker at the unique *Nde* I site located 2.5 kb downstream of the mRNA cap site. Synthetic oligonucleotides containing the κ B sequence or a related sequence (Fig. 1) were synthesized on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer by Susan Blackmon (Whitehead Institute, Oligonucleotide Synthesis Facility, Cambridge, MA). For cloning, complementary oligonucleotides were treated with kinase, hybridized, and inserted at either the *Sal* I site at position -71 or the *Xho* I site 2.5 kb downstream as indicated in the text. The total size of the plasmids was 5.1 kb.

Cell Culture, Transfections, and Enzymatic Assays. Myeloma cells (S194), pre-B-cells (38B9), or T cells (Jurkat) were maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and 50 μ M 2-mercaptoethanol. Transfections of lymphoid cells were performed by the DEAE-dextran technique (16) as modified by Grosschedl and Baltimore (17) with 10 μ g of test plasmid for 2×10^7 cells. 38B9 cells were induced with Bacto LPS (*Salmonella typhosa* 0901; Difco) at 10 μ g/ml for 40 hr. Twenty-four hours after transfection, Jurkat cells were induced with PHA

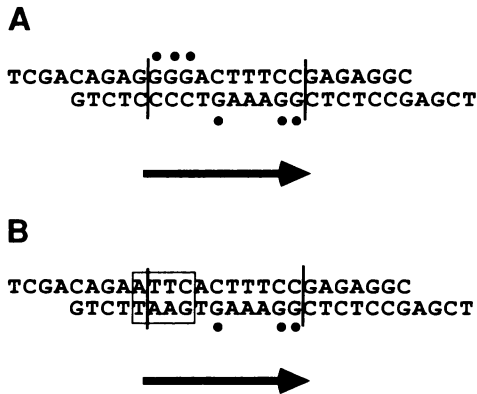


FIG. 1. Synthetic oligonucleotides. (A) κ B-containing oligonucleotide. The sequence corresponds to that found in the immunoglobulin κ light chain gene intron (positions 3937–3958) (15) with *Sal* I- and *Xho* I-compatible ends. The arrow indicates the 5'-to-3' orientation in the κ light chain gene. Filled circles indicate the guanosine residues whose methylation interferes with NF- κ B binding *in vitro* (4). (B) Mutant oligonucleotide. The sequence within the box indicates nucleotides that were altered from the sequence in A to eliminate residues important for NF- κ B binding.

(ICN Immunobiologicals, Lisle, IL) at 2 μ g/ml and PMA (Sigma) at 50 ng/ml for 18 hr. Lysates from lymphoid cells were prepared 40–44 hr after transfection and treated for 7 min at 60°C to inactivate an endogenous deacetylase activity. Chloramphenicol acetyltransferase (CAT) assays were performed by the procedure of Gorman *et al.* (18) with 100 μ g of protein for each determination. The percentage of chloramphenicol that was acetylated was determined by cutting out regions containing unreacted [14 C]chloramphenicol (New England Nuclear) and acetylated forms and quantitating the amount of radioactivity by liquid scintillation counting.

BALB/c 3T3 or HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum and transfected by the calcium phosphate coprecipitation procedure (19, 20). A mixture of 10 μ g of the test plasmid and 5 μ g of Rous sarcoma virus-conjugated β -galactosidase gene (RSV- β gal) was transfected for each experiment. After 48 hr cell lysates were prepared, and 100 μ g of protein was assayed for both CAT activity and β -galactosidase activity. The β -galactosidase levels were assayed as described (21) and used to normalize transfection efficiency.

Relative CAT activities were determined by averaging two or more independent experiments and comparing them to a basal level of activity with the control plasmid, Δ -71-*c-fos*-CAT, which contains no enhancer element. The values in the right column of Fig. 2 (relative CAT activity in 3T3 lysates) were calculated after normalizing each separate assay relative to the β -galactosidase activity. The standard deviation for relative CAT values was <20% for assays of lymphoid lysates and <30% for assays of 3T3 or HeLa lysates.

RESULTS

NF- κ B Binding Oligonucleotide Can Function as an Enhancer Element. Enhancer elements have been defined as regulatory sequences that activate transcription of a linked gene, can function in either orientation, and can act at a distance. To determine whether a short oligonucleotide containing the κ B sequence could act independently as an enhancer element, we constructed a synthetic 26-base-pair (bp) oligonucleotide including 22 bp from the κ enhancer with *Xho* I- and *Sal* I-compatible ends for cloning (see Fig. 1A). This oligonucleotide was shown to bind the nuclear

factor NF- κ B by using an electrophoretic mobility shift assay (M.L., unpublished data).

To test this oligonucleotide for enhancer properties, we inserted it into a test plasmid lacking an enhancer. The test plasmid contained a truncated *c-fos* promoter linked to the CAT gene. The *c-fos* promoter included only 71 bp upstream from the transcription start site, including one strong promoter element but lacking serum- or PMA-inducible elements (14). This promoter was chosen because it is active in a variety of cell types. The function of the κ B-containing oligonucleotide was assayed by inserting one or two copies at the 5' end of the truncated promoter or 2.5 kb downstream (Fig. 2). Test plasmids were transfected into the myeloma cell line S194, and transient expression of the CAT enzyme was used as a measure of transcriptional activity. Because the inserted oligonucleotide did not interrupt the transcribed region, differences in CAT activity were assumed to reflect differences in the rate of transcription.

The control plasmid containing the *c-fos* promoter with no inserted enhancer gave a background of \approx 0.12% conversion of chloramphenicol to its acetylated forms. Activity of plasmids containing inserts was compared to this basal level to give relative CAT activity. A single copy of the 26-bp oligonucleotide inserted at position -71 activated CAT levels about 20-fold in S194 cells (Fig. 2, lines 2 and 3). The

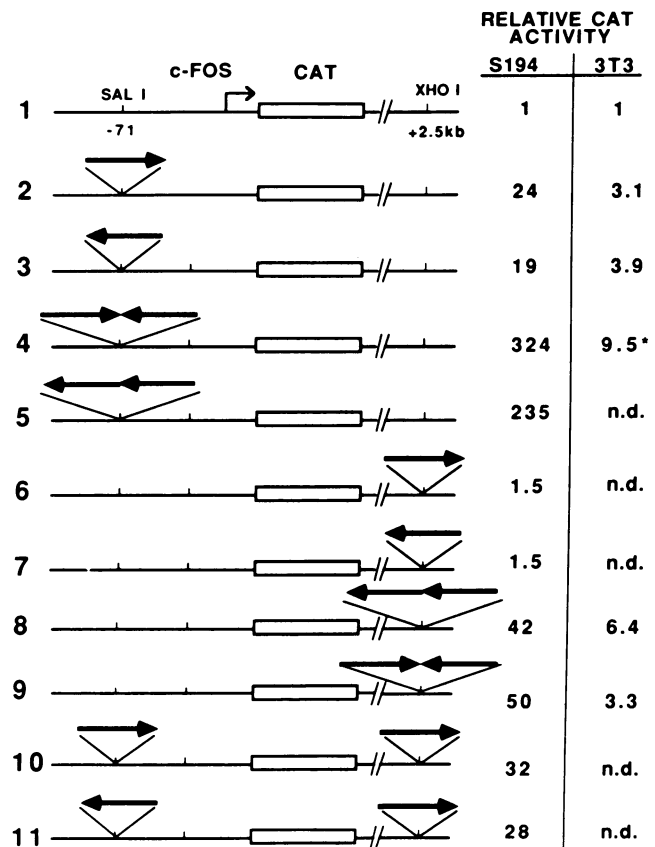


FIG. 2. Relative CAT activities directed by transfected *c-fos*-CAT plasmids with inserted κ B sites. Transfections and enzyme assays were carried out as described in text. Line 1 represents the Δ -71-*c-fos*-CAT plasmid containing no insert. The thin arrow indicates the transcriptional start site within the *c-fos* promoter. Lines 2–11 represent Δ -71-*c-fos*-CAT plasmids containing inserted κ B-containing oligonucleotides. Bold arrows indicate positions and orientations of the inserted κ B sites shown in Fig. 1. Relative CAT activity is the average of at least two independent transfections except where noted with an asterisk, which indicates that only one experiment was performed. n.d., Not determined.

κ B sequence functioned at approximately equal levels in either orientation. Interestingly, when two copies of the oligonucleotide were inserted at this position, the resulting plasmids showed a 200- to 300-fold increase in CAT activity relative to the plasmid with no enhancer (Fig. 2, lines 4 and 5). High levels of CAT activity were seen when dimers were oriented as inverted or direct repeats. The activity was much greater than would be expected if the effects of the two oligonucleotides were additive. This suggests that adjacent κ B sites collaborate to give a high level of transcriptional activation. The spacing of the NF- κ B binding nucleotides in these constructs can be deduced from previous methylation interference experiments on fragments containing this sequence (4). The guanosine residues that are important for binding (see Fig. 1A) are spaced 18 bp apart in the inverted repeat construct (Fig. 2, line 4) and 16 bp apart in the direct repeat construct (Fig. 2, line 5). A high level of transcription was achieved with either spacing.

When a single copy of the κ B site was inserted at a position 2.5 kb downstream of the start of transcription, it had little or no effect on the level of CAT activity (Fig. 2, lines 6 and 7). By contrast, two copies of the oligonucleotide inserted at this site gave a 40- to 50-fold enhancement of CAT activity (Fig. 2, lines 8 and 9). The level of enhancer activity with two κ B sites, as either direct or inverted repeats, was about the same as that observed for the intact κ enhancer. Plasmids containing one κ B site at position -71 and another at a position 2.5 kb downstream gave levels of CAT activity that were not significantly different from that seen with plasmids containing only one copy of the κ B site at the -71 position (Fig. 2, lines 10 and 11). Therefore, the presence of two widely separated κ B sites is not sufficient to generate the high level of transcription seen with adjacent sites. We have not yet determined the precise spacing requirements for the functional interaction of NF- κ B binding sites.

κ B Site Acts as a Tissue-Specific Enhancer. To determine whether the NF- κ B binding oligonucleotide acts in a tissue-specific manner, plasmids containing the κ B site were tested in a variety of cell types. In BALB/c 3T3 cells, such plasmids showed a significant increase in CAT activity when compared with the plasmid containing no enhancer (Fig. 2, right column). Thus, in these cells, the κ B site activates CAT transcription but to a much lower degree than in mature B cells. We also tested the constructs containing two κ B sites (Fig. 2, lines 8 and 9) in HeLa cells, pre-B-cells (38B9), and T cells (Jurkat). In these cell types, we observed no significant increase in CAT activity compared to plasmids lacking any enhancer (data not shown). The inactivity of the oligonucleotide in these cell types parallels the tissue specificity of the intact κ enhancer. The measurable enhancer activity of the synthetic oligonucleotide in 3T3 cells remains to be explained.

κ B Site Acts as an Inducible Enhancer. To study the role of NF- κ B binding sequences in the activation of the κ light chain gene at an earlier stage of B-cell development, we assayed the ability of this sequence to function as an inducible enhancer in tumor pre-B-cells. Test plasmids containing two copies of the κ B site were transfected into the pre-B-cell line 38B9. The intact κ enhancer is inducible with LPS in these cells (see below). Induction experiments were carried out in this cell line and not the more extensively studied 70Z/3 cell line (7) because transfection efficiency was significantly higher for 38B9 cells. The *c-fos*-CAT plasmid containing no enhancer and a plasmid containing the entire κ enhancer (470-bp *Alu* I fragment) (6) also were tested. The plasmid containing no enhancer gave the same CAT activity in LPS-treated or untreated cells (Table 1). The κ enhancer conferred 30-fold inducibility to the CAT gene. Similarly, plasmids containing two copies of the κ B site at

Table 1. κ B site acts as an LPS-inducible element in 38B9 pre-B-cells

Test plasmid	% conversion	
	- LPS	+ LPS
Δ -71- <i>c-fos</i> -CAT	<0.1	<0.1
κ -CAT	0.24	7.3
(κ B) ₂ -CAT	0.25	9.6

The values represent absolute % conversion of [¹⁴C]chloramphenicol to its acetylated forms. κ -CAT plasmid includes the 470-bp *Alu* I fragment containing the κ enhancer. (κ B)₂-CAT plasmid includes two copies of the κ B-containing oligonucleotide inserted at position -71 as shown in line 4 of Fig. 2.

the -71 position gave a 38-fold induction upon LPS treatment. Constructs containing one copy of the κ B site at the -71 position or two copies at 2.5 kb downstream gave 4- to 6-fold induction in LPS-treated 38B9 cells (data not shown). These results suggest that the κ B site acts by itself as an LPS-inducible enhancer element in pre-B-cells, and two copies have an activity as powerful as the entire κ enhancer.

To examine the function of NF- κ B binding sequences in the activation of genes in non-B-cells, we tested the ability of this sequence to act as an inducible enhancer element in T cells (Jurkat). Previous studies have shown that the NF- κ B DNA binding protein is inducible in this cell type upon treatment with PMA and PHA (7, 9). Test plasmids containing no enhancer, the HIV enhancer (22), the intact κ enhancer, or two κ B sites were transfected separately into Jurkat cells. Half of each population was treated with PMA at 50 ng/ml and PHA at 2 μ g/ml for 18 hr. The HIV-CAT plasmid, which contains the HIV long terminal repeat with both enhancer and promoter elements, showed about a 30-fold induction (Fig. 3, lanes 2 and 3) as has been observed previously (9). The construct with two κ B sites inserted in the *c-fos*-CAT plasmid at position -71 (see Fig. 2, line 4) showed a dramatic 180-fold induction (Fig. 3, lanes 6 and 7). Inducible CAT activity also was observed with plasmids containing a single copy of the κ B site at position -71 or two κ B sites at 2.5 kb downstream (data not shown). These results suggest that the NF- κ B binding sequence acts independently as a PMA-inducible enhancer element in T cells. Interestingly, the κ enhancer (470-bp fragment), which contains one κ B site and three consensus E motifs, did not confer inducibility to the CAT gene upon activation of T cells (Fig. 3, lanes 4 and 5). This marked lack of inducibility may reflect negative regulation of the κ enhancer in T cells.

A Mutant κ B Site Has No Enhancer Activity. To be certain that the enhancer activity of the 26-bp oligonucleotide was due to sequences that bind NF- κ B, we constructed a mutant oligonucleotide lacking NF- κ B binding activity. In this oligonucleotide (Fig. 1B), three nucleotides that were previously shown to be important for NF- κ B binding were altered (7). This altered sequence did not compete with the wild-type sequence for binding of NF- κ B in an electrophoretic mobility shift assay (data not shown). Two copies of the mutant oligonucleotide were inserted as inverted repeats at the *Xho* I site in *c-fos*-CAT plasmids to create a configuration identical to that on line 9 in Fig. 2. The plasmid containing mutant oligonucleotides was tested for enhancer activity in S194 cells and compared to the analogous plasmid containing NF- κ B binding sequences (Fig. 4). Whereas oligonucleotides that bound NF- κ B enhanced transcription of the CAT gene, the mutant oligonucleotides had no effect on the level of CAT activity when compared to the construct lacking an enhancer. Thus, the specific sequences that are required for NF- κ B binding are required for the enhancer function of the 26-bp oligonucleotide.

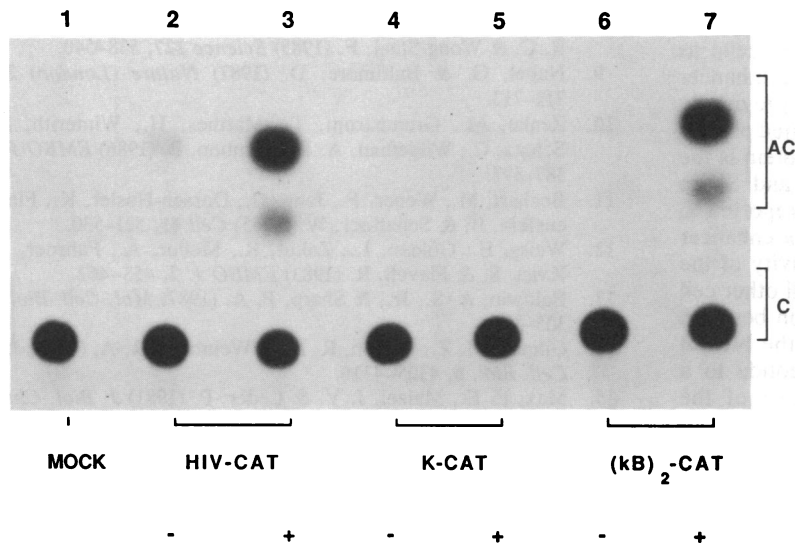


FIG. 3. CAT analysis of Jurkat cells transfected with HIV-CAT, κ -CAT, or $(\kappa B)_2$ -CAT plasmids. Jurkat cells were transfected by the DEAE-dextran procedure, and CAT analysis was performed as described in text. Induction with PMA (50 ng/ml) and PHA (2 μ g/ml) was carried out on half of the transfected populations (+ lanes) for 18 hr. Lanes: 1, mock transfected; 2 and 3, HIV-CAT (22); 4 and 5, κ -CAT plasmid, which includes a 470-bp *Alu* I fragment from the κ enhancer inserted at the *Sal* I site at -71 in *c-fos*-CAT; 6 and 7, $(\kappa B)_2$ -CAT plasmid including two κB sites inserted at -71 in *c-fos*-CAT as shown in line 4 of Fig. 2. Results of induction with PMA and PHA are shown in lanes 3, 5, and 7. The bracket marked "C" indicates unreacted substrate [14 C]chloramphenicol; the bracket marked "Ac" indicates acetylated forms.

DISCUSSION

We have shown that a short NF- κB binding sequence can act as an enhancer element independent of other motifs in the κ enhancer. As found for the entire enhancer, this sequence confers tissue specificity and inducibility to a linked gene. Monomers of this site activated transcription when positioned closely upstream of the TATA box but had no detectable function at a 2.5-kb distance downstream. By contrast, dimers of the NF- κB binding site were ≈ 10 -fold more active than monomers and showed activity at a distance.

In its natural context in the κ enhancer, a single copy of the κB site is found together with three consensus E motifs. Mutation of the κB site abolishes the activity of the enhancer (6), identifying it as a crucial enhancer element. The ability of this site to act independently of others suggests that the E motifs are not necessary to enhancer function, although both

mutational studies (6) and our present data show that the E motifs contribute to enhancer activity. This contribution is evident when the activity of the κB site is compared to that of the entire κ enhancer in the downstream position. A single κB site by itself is not sufficient for enhancer activity at a distance. However, two copies of the κB site are as active as the entire enhancer.

Our observation that dimers of the κB site enhance transcription of a linked gene 10-fold better than a monomer does is consistent with previous results on the oligomerization of short protein-binding segments from other enhancers. Gerster *et al.* (23) showed that a fragment from the immunoglobulin heavy chain gene enhancer containing an "octamer" motif and an E motif ("footprint" region) functions as a lymphoid-specific enhancer when multimerized but also showed that a single copy had only a marginal effect on transcription. In addition, multimers of short protein-binding sequences from the SV40 enhancer have been shown to act as cell type-specific enhancer elements (24, 25). It is interesting to compare our results to those of Schirm *et al.* (24) and Ondek *et al.* (25) on the oligomerization of an SV40 element that includes an NF- κB binding site. Multimers of the "core C" element from SV40, containing a κB site and the core consensus sequence TGGAAAG, gave enhancer activity in many lymphoid and nonlymphoid cell lines. This activity could be due to proteins binding at the κB site, the SV40 core, or other sequences in this region. Our oligonucleotide from the κ enhancer showed preferential activity in mature B cells. We showed by analysis of a mutant oligonucleotide that the NF- κB binding site is the only sequence within our 26-bp oligonucleotide that functions as an enhancer. Thus, the NF- κB binding site by itself acts in a lymphoid-specific fashion but, when combined with an overlapping element such as the SV40 core consensus, the resulting composite sequence is active in many cell types. Unique patterns of tissue-specific or stage-specific gene expression appear to be achieved through the combinatorial effects of different trans-acting factors, each having a distinct activity.

We observed a synergistic activity of adjacent κB sites. This may be the result of cooperative binding of NF- κB protein to the duplicated sites on the DNA or to a more productive interaction of the bound NF- κB protein with other proteins of the transcriptional apparatus. In the context of the SV40, HIV, or cytomegalovirus enhancers, the κB site naturally occurs in two or more copies, and this may account for the high activity of these enhancers in certain cell types.

Interestingly, we found that the NF- κB binding sequence activates transcription in non-B-cells. In Jurkat cells induced

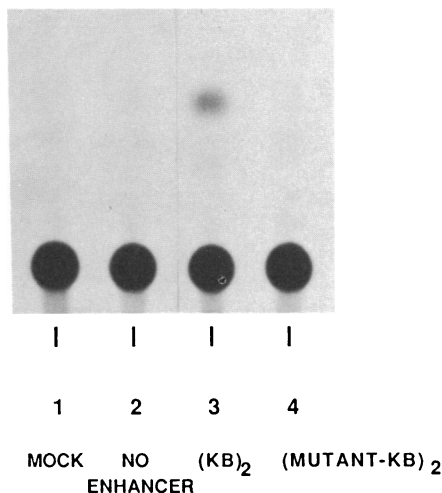


FIG. 4. Effect of mutation of the κB site on the enhancer activity of the synthetic oligonucleotide. S194 myeloma cells were transfected by the DEAE-dextran procedure, and extracts were assayed for CAT activity as indicated in text. Extracts were from untransfected cells (lane 1), cells transfected with Δ -71-*c-fos*-CAT plasmid (no enhancer) (lane 2), cells transfected with Δ -71-*c-fos*-CAT with two κB sites inserted as inverted repeats at 2.5 kb from the transcriptional start site as shown in Fig. 2, line 9 (lane 3), and cells transfected with Δ -71-*c-fos*-CAT with two mutant κB sites (see Fig. 1B) inserted as inverted repeats at 2.5 kb from the transcriptional start site (lane 4).

with phorbol esters, activity is probably due to a protein that is similar or identical to the NF- κ B found in B cells as defined by binding assays (7, 9). The low level of enhancer activity in 3T3 cells may be due to a low and previously undetected level of NF- κ B protein or to some related protein that binds this sequence. A candidate for this protein is the nuclear factor, H2TF1, described by Baldwin and Sharp (13), which is present in 3T3 cells and has been reported to interact at a low level with the κ B site from the κ enhancer (26). This factor may be responsible for the activity of the NF- κ B binding oligonucleotide in 3T3 cells. In all other cell types examined, we found a complete correlation between the activity of the κ B site and the presence of the NF- κ B factor. Thus, the binding of a short oligonucleotide to a single nuclear factor, NF- κ B, accounts for most of the properties of the intact κ enhancer, including lymphoid-specificity and developmental activation during B-cell ontogeny.

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